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Pathogenesis of Lassa Virus Infection in Guinea Pigs

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A rodent model for human Lassa fever was developed which uses inbred (strain 13) and outbred (Hartley) guinea pigs. Strain 13 guinea pigs were uniformly susceptible to lethal infection by 2 or more PFU of Lassa virus strain Josiah. In contrast, no more than 30% of the Hartley guinea pigs died regardless of the virus dose. In lethally infected strain 13 guinea pigs, peak titers of virus (10^7 to 10^8 PFU) occurred in the spleen and lymph nodes at 8 to 9 days, in the salivary glands at 11 days, and in the lung at 14 to 16 days. Virus reached low titers (10^4 PFU) in the plasma and brain and intermediate titers in the liver, adrenal glands, kidney, pancreas, and heart. In moribund animals, the most consistent and severe histological lesion was an interstitial pneumonia. In contrast, the brain was only minimally involved. The immune response of lethally infected strain 13 guinea pigs, as measured by the indirect fluorescent antibody test, was detectable within 10 days of infection and was similar in timing and intensity to the fluorescent antibody test response of both lethally infected and surviving outbred animals. In contrast to the fluorescent antibody response, neutralizing antibody developed late in convalescence and was thus detected only in surviving outbred guinea pigs. The availability of a rodent model for human Lassa fever in uniformly susceptible strain 13 guinea pigs should facilitate detailed pathophysiological studies and efficacy testing of antiviral drugs, candidate vaccines, and immunotherapy regimens to develop control methods for this life-threatening disease in humans.

The development of animal models for human Lassa fever would facilitate the development of effective treatment and immunization regimens and might provide insight into the pathogenesis of this life-threatening disease. We recently described Lassa virus infection in rhesus monkeys and the effective treatment of monkeys with the antiviral drug ribavirin (3). Previously, other investigators have described Lassa virus infections in humans (10) and squirrel monkeys (9). We have extended these descriptive studies to include cynomolgus, African green, and capuchin monkeys (P. B. Jahrling, manuscript in preparation). Studies with primates are extremely expensive, however, especially when conducted in maximum containment (P4) laboratories, and the availability of a small rodent model for lethal Lassa virus disease approximating the human disease would be useful. The use of outbred Hartley strain guinea pigs was explored previously (9); the authors concluded that the disease process in outbred guinea pigs was too different from human Lassa fever to warrant extensive investigation. This conclusion deserves reexamination, however, in light of our recently reported observations (4) that inbred strain 13 guinea pigs develop a uniformly lethal infection resembling human Lassa fever after infection with an adapted strain of Pichinde virus, an arenavirus distantly related to Lassa virus (4), whereas outbred guinea pigs are more resistant. These observations prompted us to reexamine Lassa virus infection in guinea pigs, comparing strain 13 and outbred animals to gain insight into the determinants for susceptibility and resistance to Lassa infection and to develop a uniformly susceptible model for testing treatment and immunization regimens. We report here the exquisite sensitivity of strain 13 guinea pigs to lethal Lassa virus infection, the replication of virus in target tissues, the development of histological lesions, and humoral immune responses to Lassa virus infection.

MATERIALS AND METHODS

Preparation of virus stocks and virus assay. Lassa virus strain Josiah was isolated in 1976 from the serum of a 40-year-old man in Sierra Leone, Africa (11). This isolate was passaged four times at low multiplicity (1 PFU per 100 cells) in monolayer cultures of Vero cells (an African green monkey kidney cell line) and grown to confluency in 75-cm^2 flasks containing 5×10^6 cells. This stock virus suspension (3), stored at −70°C, contained 2.5×10^7 PFU/ml.

All of the infectious Lassa virus assays were performed by counting PFU on Vero cell culture monolayers grown in 10-cm^2 wells of plastic plates and maintained under medium containing 1% agarose, basal medium (Eagle) with Earle salts, HEPES (*N*-2-

hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (25 mM), and 2% fetal calf serum as described previously (3). Cells were incubated at 36°C for 5 days in a humidified atmosphere containing 5% CO_2. After the incubation, 2 ml of neutral red diluted in Puck saline A to a final concentration of 1:6,000 was added to the cells; plaques were counted after an additional incubation period of 18 to 24 h.

Inoculation of guinea pigs and harvest of tissues. All manipulations of Lassa virus, infected animals, and unfixed samples obtained from infected animals were conducted within the maximum biological containment (P4) facilities at the USAMRIID. Inbred male strain 13 guinea pigs weighing 350 to 500 g were obtained from the Institute's colony; male strain 2 guinea pigs (450 to 600 g) were obtained from Crest Caviary (Covina, Calif.), and outbred male Hartley strain guinea pigs (400 to 500 g) were from Buckberg Farms (Tomkins Cove, N.Y.). All of the guinea pigs were inoculated subcutaneously (s.c.) with virus suspensions diluted in Eagle minimal essential medium with Earle salts plus 10% fetal calf serum. Virus doses were varied as stated below. Plasma samples were obtained from ether-anesthetized guinea pigs bled repetitively at 2- to 3-day intervals from the retroorbital sinus into heparinized syringes with 25-gauge needles. Guinea pigs killed to provide infected tissues and large volumes of plasma were anesthetized with chloroform and bled from the heart into heparinized syringes. Tissues were removed aseptically, and a portion of each tissue was homogenized in minimal medium plus 10% fetal calf serum with mortars and pestles and Alundum. The 10% (wt/vol) clarified suspensions were assayed for infectious virus. A second portion of each tissue was prepared for immunofluorescence examination by being embedded in a polyethylene glycol compound (O.C.T.; Ames Co., Elkhart, Ind.), frozen on powdered dry ice, and stored in a freezer at −70°C until cut in a cryostat. The remaining tissue portions were fixed in 10% neutral buffered Formalin for the preparation of standard histological sections and stained with hematoxylin and eosin. Heparinized blood samples were diluted with Unopettes (Becton, Dickinson & Co., Rutherford, N.J.), and total leukocyte counts were obtained with a hemacytometer. Leukocytes were further differentiated by observation of Wright-Giemsa-stained blood films. Transaminase concentrations in the plasma were measured spectrophotometrically with Statzyme kits (Worthington Diagnostics, Freehold, N.J.) for serum glutamic oxaloacetic transaminase (SGOT).

Immunofluorescence. For the detection of Lassa virus antigens in tissue sections, a direct fluorescent antibody test was used as described previously (3). In brief, hyperimmune serum was prepared by repeated inoculation of rhesus monkeys with Lassa virus strain Josiah (6.1 $\log_{10}$ PFU s.c.). Immunoglobulins were precipitated from the serum with methanol and conjugated with fluorescein isothiocyanate. Frozen tissue sections 6- to 10-nm thick were fixed in acetone at room temperature for 10 min, washed in phosphate buffered saline (PBS; pH 7.4) for 10 min, and then immediately flooded with conjugate and incubated for 30 min in a humidified chamber at room temperature. The sections were then washed with PBS for 10 min and mounted under PBS-glycerol. Slides were examined with a Leitz Dialux immunofluorescence microscope equipped with dry objectives and an incident 50 W mercury light source. Controls routinely included stained tissues from uninfected guinea pigs and infected tissues treated with fluorescent conjugates prepared against a serologically unrelated virus (Venezuelan encephalitis virus). During standardization, inhibition (or blocking) controls which used unconjugated monkey anti-Lassa serum, followed by the direct conjugate, were included. All of the controls were unremarkable, exhibiting no fluorescence which could be confused with that observed in infected tissues stained with the Lassa-specific conjugate.

Antibody assays. The indirect fluorescent antibody (IFA) test was performed by the procedure of Peters et al. (8) with slight modifications (2). In brief, Vero cells infected with Lassa virus or uninfected control cells were dried onto circular areas of Teflon-coated slides (Cel-Line Associates, Minatola, N.J.), fixed in acetone, and treated with test serum diluted in PBS. Each test included two positive control guinea pig sera with anti-Lassa IFA titers of 1:80 and 1:5,120, respectively, to insure standardization of the assay. After being incubated for 30 min at room temperature, the slides were washed in PBS for 30 min, flooded with fluorescein-conjugated goat anti-guinea pig gamma globulin, incubated and washed again, mounted, and examined. The endpoint was the highest dilution of serum which produced definite granular fluorescence of the cytoplasm of infected cells. Since residual infectious virus could routinely be recovered from these slides even after staining, all of the slides were observed under P4 containment conditions.

Neutralizing antibody (N-antibody) titers were measured in a plaque reduction test by the constant serum–varying virus format, the rationale for which will be detailed elsewhere (Jahrling, manuscript in preparation). In brief, all dilutions were performed in a neutralization test medium (NTM) composed of Hanks balanced salt solution, HEPES buffer (25 mM), and 10% freshly obtained normal guinea pig serum as a complement source. Challenge virus was the Lassa virus strain Josiah preparation described above diluted initially 1:10 in NTM, in which it had a titer of 6.2×10^6 PFU/ml. To test the neutralizing capacity, we diluted the test sera (freshly obtained or stored frozen at −70°C until used) 1:10 in NTM and divided the sera into a series of six aliquots (0.9 ml each). Challenge virus was then serially diluted in tenfold increments in the NTM containing test serum, and the reaction mixtures were incubated at 37°C for 1 h. As a control, normal guinea pig serum was substituted for immune guinea pig test serum. After incubation, the reaction mixtures were assayed for residual infectivity (i.e., PFU) on Vero cells as detailed above. Wells containing 10 to 100 PFU were counted. N-antibody activity was expressed as a $\log_{10}$ neutralization index (LNI), calculated by the formula LNI = $\log_{10}$ [(PFU in control) − (PFU in test serum)].

RESULTS

Lassa virus strain Josiah infected and killed all of the strain 13 guinea pigs inoculated s.c. with 2 PFU or more (Table 1); tenfold less virus (0.2 PFU s.c.) killed 4 of 10. The surviving strain 13 guinea pigs did not seroconvert by the IFA test, nor did they resist backchallenge, suggesting

TABLE 1. Infectivity and lethality of Lassa virus strain Josiah for guinea pigs inoculated s.c.

Guinea pig strain	Inoculum (PFU)	No. dead/no. tested (%)	Mean day of death (range)	Sero-converted[a]	Resisted rechallenge[b]
Strain 13	240,000	5/5 (100)	15.5 (11–17)		
	2,400	15/15 (100)	17.4 (16–19)		
	24	10/10 (100)	18.4 (17–24)		
	2	10/10 (100)	17.1 (15–19)		
	0.2	4/10 (40)	20.0 (18–22)	0/6	0/6
	0.02	1/10 (10)	18	0/9	0/9
Strain 2	2,400	10/10 (100)	18.1 (15–20)		
Outbred Hartley	240,000	9/30 (30)	17.0 (15–19)	21/21	21/21
	2,400	6/20 (30)	17.0 (15–19)	14/14	14/14
	24	6/19 (32)	16.8 (15–19)	13/13	13/13
	2	10/30 (30)	17.6 (16–19)	20/20	20/20
	0.2	4/25 (16)	18.0 (17–19)	9/21	13/21

[a] Considered positive if IFA titers were ≥10 on days 45 to 50.
[b] Lived 45 days or longer after s.c. inoculation of 2,400 PFU of Lassa virus strain Josiah.

that inapparent infections did not occur in the strain 13 guinea pigs. Likewise, all of the 10 strain 2 guinea pigs inoculated with a moderate Lassa virus dose (2,400 PFU s.c.) were lethally infected. In contrast, outbred Hartley strain guinea pigs were relatively resistant; approximately 30% of them died at all doses of 2 PFU or more tested (Table 1). Inapparent infections occurred, since all of the surviving guinea pigs inoculated with 2 PFU or more seroconverted and all resisted backchallenge. These results suggested that the strain 13 guinea pig would serve as an excellent model for analyzing the pathogenesis of lethal Lassa virus infection. In contrast, outbred guinea pigs would be useful for producing Lassa immune serum.

To characterize Lassa virus infections of guinea pigs in more detail, we compared virus titers among groups of 15 guinea pigs inoculated with 3.4 $\log_{10}$ PFU and bled repetitively until day 21 or until they died (Fig. 1). Viremia in strain 13 guinea pigs developed more rapidly and reached higher titers than it did in either the lethally infected or surviving outbred groups. On days 4 and 6, viremias for strain 13 guinea pigs were significantly ($P < 0.05$) higher than for either outbred group. Viremia persisted at the highest titer in strain 13 guinea pigs and at somewhat lower titers in lethally infected outbred guinea pigs. In the surviving outbred animals, viremias declined to undetectable titers by day 21. These data suggest that viral replication proceeded more efficiently in strain 13 than it did in the outbred animals and that host response mechanisms did not effectively restrict viral replication in the strain 13 guinea pigs or in outbred guinea pigs with an ultimately fatal infection.

Humoral antibodies to Lassa virus were measured for all three groups. Despite differences in the viremia titer curves, the antibody responses measured by IFA were similar in both timing and magnitude among the groups. IFA and LNI antibody responses in the outbred guinea pigs which survived infection are compared in Fig. 2. IFA titers reached 1:40 or more by day 10 and 1:320 to 1:640 by day 16. In surviving guinea pigs, viremia declined to undetectable titers by day 21. However, N-antibody was not detectable on days 21, 28, or 32. N-antibody titers were first detected on day 45 and increased with time for 180 days, the last time tested.

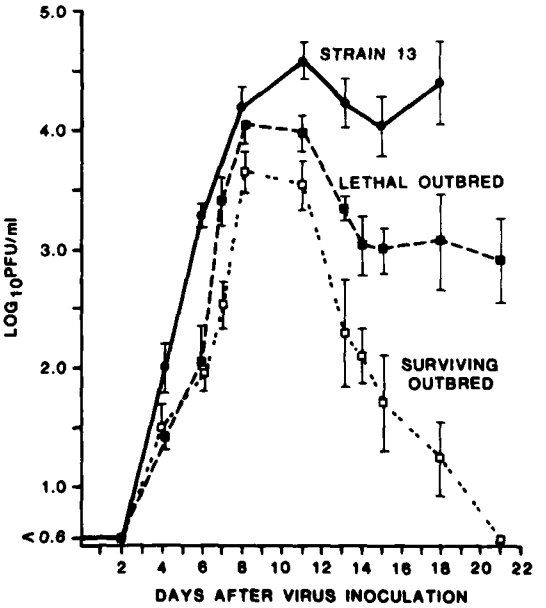

FIG. 1. Development of Lassa viremia in lethally infected strain 13 (●) and outbred (■) guinea pigs and in surviving outbred (□) guinea pigs inoculated s.c. with 3.4 $\log_{10}$ PFU. Points are geometric means (± standard error) of 15 guinea pigs.

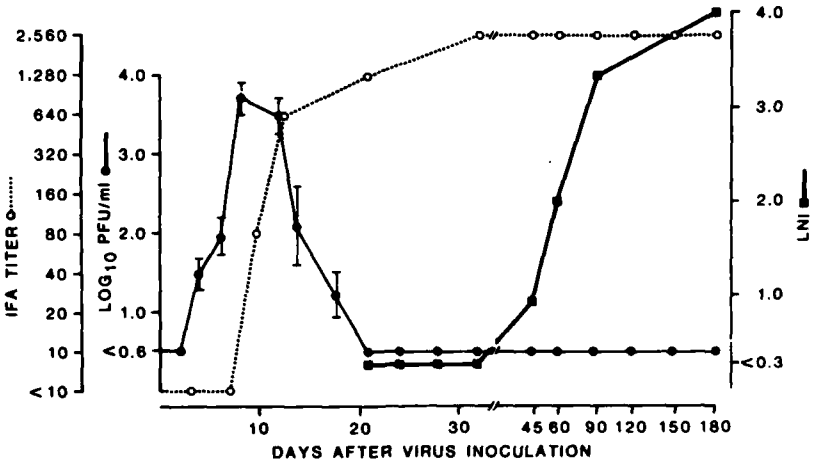

FIG. 2. Development of Lassa viremia (●) IFA responses (○), and N-antibody responses (■) in surviving outbred guinea pigs inoculated s.c. with 3.4 $\log_{10}$ PFU.

Further descriptive studies of fatal Lassa virus pathogenesis were initiated with strain 13 guinea pigs, which were uniformly killed by Lassa virus infection. Leukopenia was not severe; even in moribund animals, the average total leukocyte count was 6,200 cells per mm^3. However, differential counts revealed absolute lymphopenia which was offset by neutrophilia. Hemoglobin concentrations increased by day 8 but receded to near normal values by day 16 (Fig. 3). SGOT activity increased steadily throughout the disease course but never exceeded 200 IU/liter.

To obtain additional insight into the tissue tropisms and histological lesions produced by Lassa virus, we inoculated guinea pigs with 2,400 PFU s.c. Groups of infected animals were killed at 3- to 4-day intervals to obtain tissues for infectivity titrations; in addition, eight guinea pigs were killed when moribund on days 16 to 18 to correlate the presence of infectious virus with the distribution of viral antigens and histopathological lesions in moribund animals. Infectivity data for tissues obtained in the sequential study (Fig. 4) suggested that the virus replicated in all of the extraneural tissues examined but not in the brain. High titers of virus occurred early in the lymph node, spleen, salivary gland, pancreas, and lung; virus concentrations in other tissues increased more slowly. In the group of eight moribund guinea pigs examined 16 to 18 days after infection, the highest titers of virus were recovered from the lung (Fig. 5). As judged by immunofluorescence, all of the eight lungs examined contained viral antigens. The spleen contained the second highest concentration of virus, followed by the pancreas, lymph node, adrenals, kidney, salivary gland, liver, and heart (Fig. 5). Virus recovered from the brain was of a sufficiently low titer to be attributable to the blood-borne virus. High concentrations of viral antigens were readily detected by immunofluorescence in all lung (Fig. 6A), spleen, pancreas, kidney (Fig. 6B), and salivary gland specimens examined but less frequently detected in liver (five of eight), heart (two of eight), and brain (two of eight) samples.

The distribution and degree of histological damage observed in these tissues was less than would have been predicted on the basis of the infectivity and immunofluorescence data. Lung

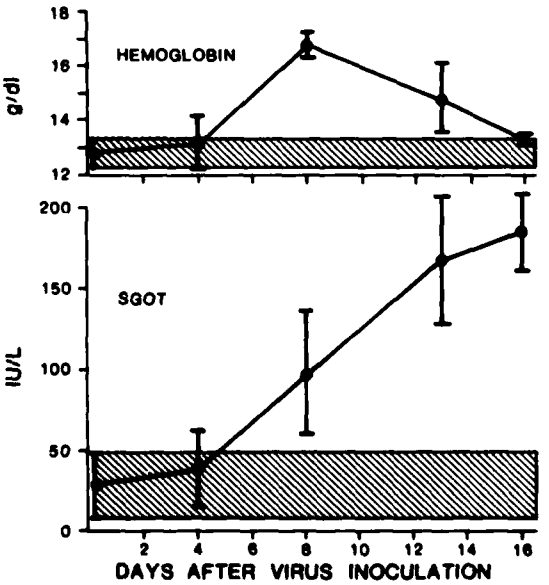

FIG. 3. Hemoglobin concentrations in blood and SGOT activity in the sera of strain 13 guinea pigs inoculated s.c. with 3.4 $\log_{10}$ PFU of Lassa virus. Points are arithmetic means (± standard error) of 10 guinea pigs.

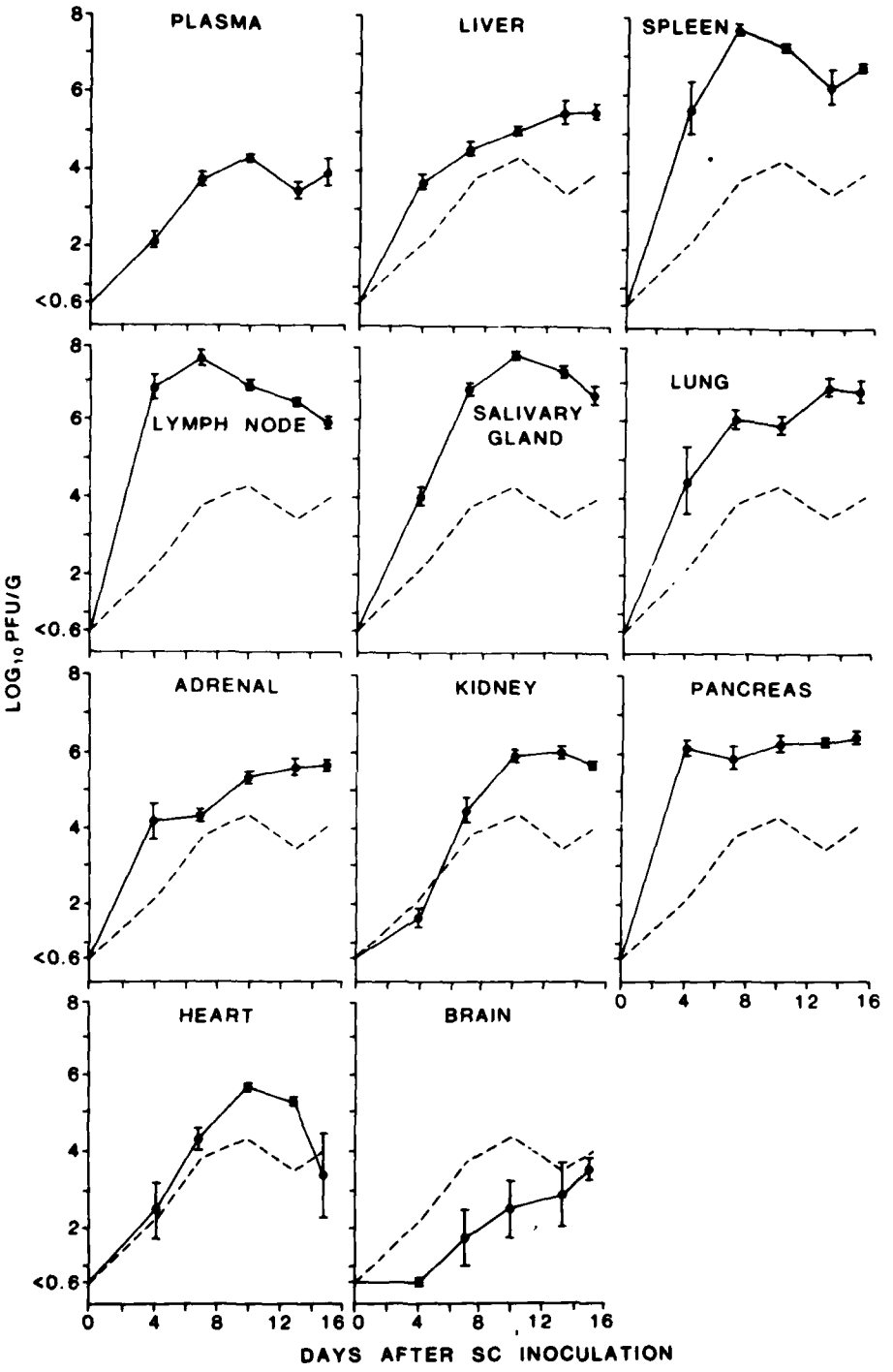

FIG. 4. Infectious Lassa virus concentrations recovered from serum and tissues of strain 13 guinea pigs inoculated s.c. with 3.4 $\log_{10}$ PFU. Points are geometric means (± standard error) of five guinea pigs. The viremia curve (----) is superimposed on each tissue curve (●—●) to facilitate comparison.

tissue from all eight guinea pigs revealed areas of interstitial pneumonia, which varied in degree from mild to moderate (Fig. 6C). Four of eight kidney sections displayed acute necrotizing nephritis, a lesion complicated in two of the four instances by the presence of bacterial colonies (Fig. 6D). Likewise, five of eight spleen sections contained necrotizing lesions of varying severity

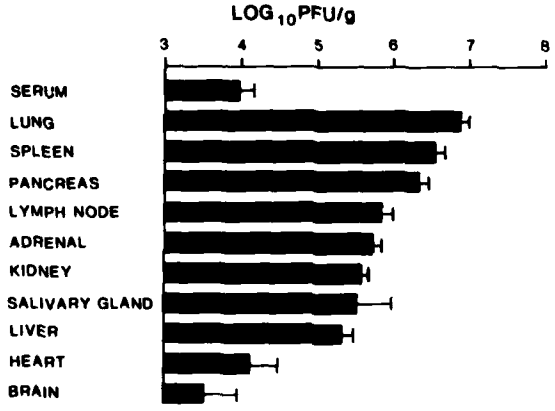

FIG. 5. Infectious Lassa virus concentrations recovered from sera and tissues of moribund strain 13 guinea pigs obtained 16 to 18 days after s.c. inoculation with 3.4 $\log_{10}$ PFU. Titers are geometric means (± standard error) of eight moribund guinea pigs. These are the same animals whose tissues were examined by immunofluorescence for viral antigens and by light microscopy for histological lesions.

involving bacteria. Mild myocarditis was observed in four of eight guinea pigs, and minimal hepatitis accompanied by diffuse fatty change was found in five. Although the virus clearly replicated in the adrenal and salivary glands and pancreas, no remarkable lesions were seen in these tissues. Brain tissues from two guinea pigs were examined but contained no lesions.

DISCUSSION

The studies reported here suggest that guinea pigs are a potentially useful model for elucidating the pathogenesis of Lassa virus infection and for developing effective treatment and protection regimens. Strain 13 guinea pigs were uniformly killed by Lassa virus infection, whereas outbred Hartley strain animals were relatively resistant. Thus, strain 13 guinea pigs are preferable to outbred animals for studying the pathogenesis of acutely lethal Lassa virus infection and for assessing vaccine and treatment regimens. Conversely, outbred guinea pigs offer an opportunity to generate convalescent antisera, examine the natural basis for fatal versus nonfatal disease, and search for late sequelae (if any) of these infections. These observations are similar to those we reported for Pichinde virus, which, like Lassa, uniformly killed inbred strain 13 guinea pigs but killed less than half of outbred animals regardless of the dose (4). The uniform susceptibility of strain 13 guinea pigs and the dose-independent pattern of resistance among outbred animals suggest a genetic basis for the resistance or susceptibility of guinea pigs to both Pichinde and Lassa virus infections. These factors include humoral and cellular immune responses, whose roles in the resolution or exacerbation of disease remain to be defined.

N-antibody is clearly protective when administered in adequate concentrations to inbred guinea pigs (manuscript in preparation). However, it is difficult to make a case for N-antibody as a critical factor in the resolution of acute Lassa virus infections in outbred guinea pigs since N-antibody concentrations were not detectable until several weeks after the viremia had subsided (Fig. 2). Although antigen concentrations in tissues are massive and could conceivably remove N-antibody from the circulation, passively administered N-antibody is not cleared from the circulation of infected guinea pigs any more quickly than it is from uninfected controls (manuscript in preparation). Differences in susceptibility are probably also unrelated to the early IFA response, since these were similar in both timing and magnitude for inbred and outbred animals. Furthermore, early convalescent sera contained high concentrations of antibody as measured by IFA but conferred no demonstrable protection to infected guinea pigs (manuscript in preparation), suggesting that the IFA response is of little consequence in resolving acute Lassa virus infections. Differences in cellular immune responses, including quantitative differences in reticuloendothelial cell function, may explain differences in susceptibility. The availability of a model which uses inbred guinea pigs should facilitate adoptive cell transfer studies to approach these problems. Differences in the susceptibility of guinea pig strains may also depend on genetically determined differences in cellular susceptibility to productive viral infection or perhaps to defective interfering particle generation. The existence of increased numbers of target cells in susceptible animals may also be a factor. In an analogous study (1), the increased susceptibility of MHA strain hamsters as compared with LVH hamsters was correlated with increased numbers of natural killer cells in the spleens of MHA hamsters; natural killer cells appeared to be critical targets for Pichinde virus replication.

The recovery of infectious Lassa virus from all of the extraneural tissues examined, in concentrations which suggest viral replication, was similar to the tissue distributions reported by us for Lassa virus in rhesus monkeys (3) and Pichinde virus in guinea pigs (4), and by others for Lassa virus in humans (10) and guinea pigs (9). The presence of high concentrations of infectious Lassa virus in the spleen and lymph nodes early in the disease course suggests early replication of the virus in these tissues and is consistent with the reticuloendothelial tropism reported for other arenaviruses in animal models (5, 6). In

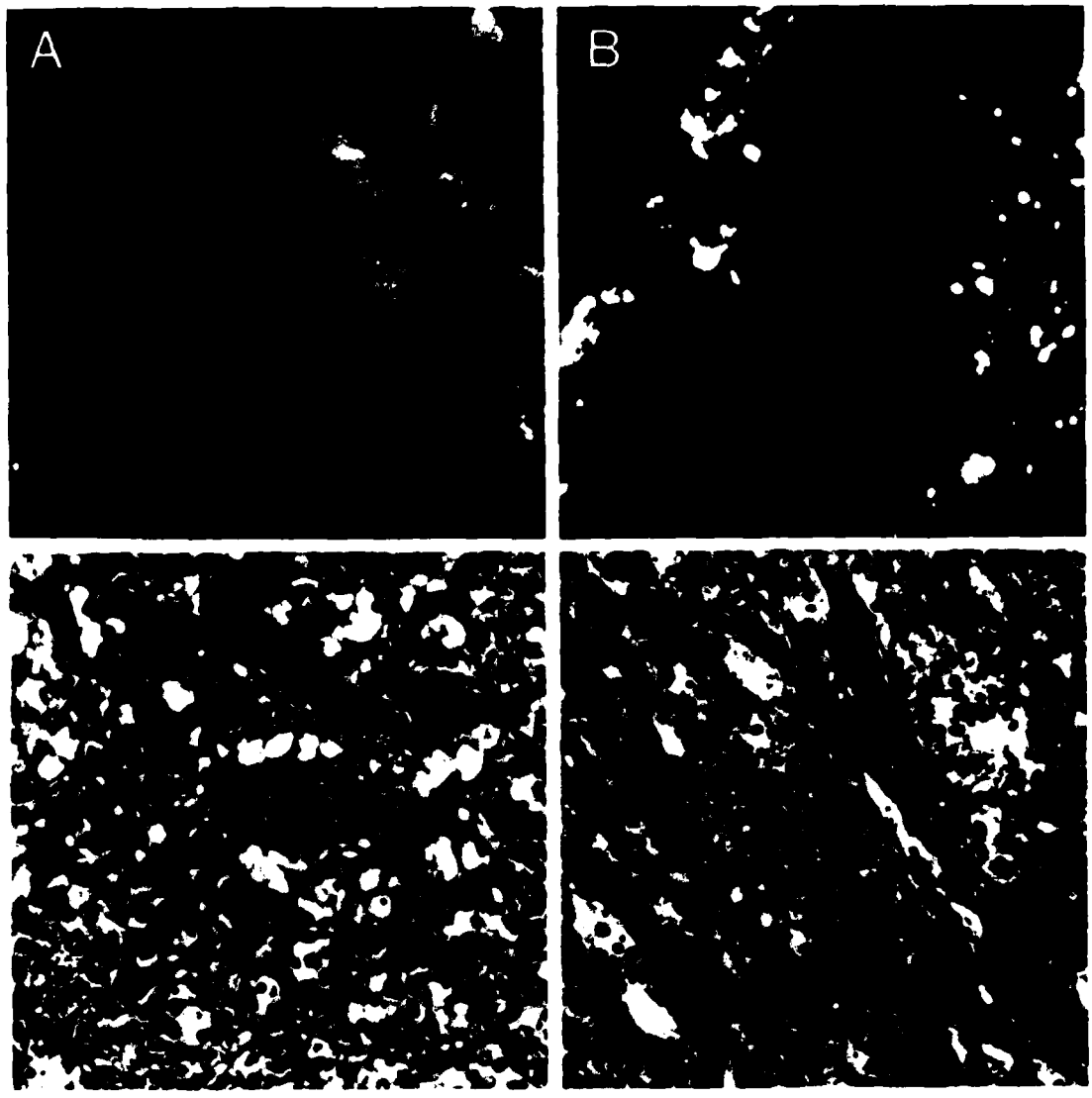

FIG. 6. (A) Lassa viral antigens in thickened alveolar septae of lung (direct fluorescent antibody test). (B) Lassa viral antigens associated with renal tubules (direct fluorescent antibody test). Glomeruli contained no viral antigens. (C) Interstitial pneumonia, moderately severe, accompanied by edema and hemorrhage (hematoxylin and eosin stain). (D) Acute necrotizing nephritis with associated bacterial colonies; same kidney as shown in Fig. 6C (hematoxylin and eosin stain). (×440)

the present study, central nervous system involvement appeared to be minimal; immunofluorescent staining of brain sections revealed infection of occasional, isolated cells in association with small blood vessels, which could be of pathological significance especially in animals which survive the acute, viscerotropic infection. Lassa virus concentrations in guinea pig tissues were slightly lower than those we reported for rhesus monkey tissues (2); for most tissues, this difference was only about 1 $\log_{10}$ PFU/g. However, of the guinea pig tissues tested, the lungs contained the highest concentration of virus, 6.9 $\log_{10}$ PFU/g, which was slightly higher than that reported for monkey lung (3). Conversely, the liver did not appear to be a major site of Lassa virus replication in guinea pigs (5.1 $\log_{10}$ PFU/g) as compared with monkeys, in which the liver contained the highest concentration of virus (7.6 $\log_{10}$ PFU/g) of all of the tissues tested. Virus titers in the tissues obtained in the sequential study (Fig. 5), the relatively modest SGOT elevations (Fig. 3), and the immunofluorescence study all confirmed that the liver is not a major target of Lassa virus replication in guinea pigs. Thus, with respect to hepatotropism the Pi-

chinde guinea pig model (4) more closely resembles human and primate Lassa virus infection than do the Lassa guinea pig models reported herein and previously (9).

Histological lesions were generally mild. Interpretation of the lesions was complicated by the presence of bacterial colonies, which may have been a significant factor in the pathogenesis of this disease. Terminal bacteremia has occasionally been documented for human Lassa fever patients (J. B. Frame, personal communication) and more frequently in cynomolgus, rhesus, and African green monkeys experimentally infected with Lassa virus (P. B. Jahrling, unpublished data). Bacteremia may be a frequent rather than incidental consequence of Lassa virus infection of the lymphoreticular organs, leading to reticuloendothelial cell dysfunction and possibly to a generalized immunosuppression. However, in guinea pigs the severe histological lesions, including necrotizing hepatitis and interstitial pneumonia, usually associated with Lassa virus infection in primates (2; R. T. Callis, P. B. Jahrling, and A. DePaoli, submitted for publication) occurred infrequently or were of minimal severity.

The replication of Lassa virus in tissues showing little or no histological damage or inflammation is a recurrent theme (3, 9). Disease may result as a consequence of functional impairment of infected cells, which appear histologically normal but are inhibited from performing the specialty functions of differentiated cells (7). For example, the infection of adrenocortical cells, which contain high concentrations of Lassa antigens as visualized by immunofluorescence, may result in an imbalance in corticosteroid metabolism which could contribute to death. The pathophysiology of arenavirus disease remains to be studied in more detail. Insight critical to the effective treatment of Lassa virus infections may be obtained by studying the biochemical and hematological changes in blood, hepatic, and cardiovascular functions and capillary permeability changes in various animal models. The increased hemoglobin concentration observed (Fig. 3) is presently unexplained but is consistent with the hemoconcentration associated with other human hemorrhagic fever virus infections. Our initial pathophysiological studies will focus on Pichinde in guinea pigs (4), since this virus poses a reduced biohazard as compared with Lassa virus. However, critical pathophysiological studies will be repeated with Lassa virus in guinea pigs. In the interim, we anticipate that strain 13 guinea pigs will be useful for testing antiviral drugs, candidate vaccines, and immunotherapy regimens.

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